

Notes & Tips

An overlay technique for postelectrophoretic analysis of proteinase spectra in complex mixtures using *p*-nitroanilide substrates[☆]

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A simple method with improved sensitivity for the detection of different groups of proteinases in complex mixtures separated by polyacrylamide electrophoresis and overlaid with a nitrocellulose membrane impregnated with specific *p*-nitroanilide (pNA)¹ substrates is described. A drawback of the direct testing of proteinase activity in a polyacrylamide gel with chromogenic peptide substrates is the high degree of diffusion of low-molecular-mass reaction products. Ohlsson et al. [1] proposed the transfer of proteinases to a nitrocellulose membrane after electrophoresis in a 1% agarose gel with subsequent detection of activity after incubation with pNA substrate solutions. Later this technique was transferred to SDS–PAGE by means of electrotransfer and was actively used for the characterization of digestive proteinases in insects [2,3]. However, conservation of proteinase activity during electrotransfer is time and

temperature dependent, and enzyme activity can be lost after 1 h of electrotransfer, even for relatively stable serine proteinases [4]. In this study analysis by an overlay of nitrocellulose impregnated with pNA substrates on a polyacrylamide gel was more effective than electrotransfer for the preservation of proteinase activity in complex mixtures. Instead of SDS–PAGE, native PAGE at neutral pH in Hepes–imidazole buffer [5], which can be performed in two directions, was used for anionic and cationic proteins. This electrophoretic method retained proteolytic activity, especially for cysteine proteinases, which may be reduced or even irreversibly lost in denaturing conditions and/or alkaline pH in SDS–PAGE [6]. Specific inhibitors used for characterization of proteinases are added to proteinase mixtures typically before or after PAGE [4]. We demonstrate that the addition of inhibitors after PAGE was more reliable and excluded the possible breakdown of proteinase–inhibitor complexes during electrophoresis. The methods were developed with preparations of digestive proteinases from the yellow mealworm (*Tenebrio molitor*) larval anterior midgut (AM), where the pH of contents is 5.6 and cysteine proteinases are predominant, and the posterior midgut (PM), where the pH of contents is 7.9 and serine proteinases are predominant [7].

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¹ Abbreviations used: AM, anterior midgut; BzRpNA, *N*_α-benzoyl-DL-arginine *p*-nitroanilide; DTT, dithiothreitol; E-64, L-trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane; GlpAALpNA, pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide; GlpFAPNA, pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide; PM, posterior midgut; PMSF, phenylmethylsulfonyl fluoride; pNA, *p*-nitroanilide; STI, soybean Kunitz trypsin inhibitor; TLCK, *N*_α-tosyl-L-lysine chloromethyl ketone.

Materials and methods

Proteinases. Fifty excised AM and PM of fourth instar *T. molitor* larvae were homogenized in a glass homogenizer containing 350 μl of ice-cold deionized

water and centrifuged for 10 min at 15,000g; the supernatants were stored at -70°C .

Substrates. N_{α} -Benzoyl-DL-arginine pNA (BzRpNA) was from Fluka (Switzerland), and all other substrates were synthesized and provided by Dr. Irina Yu. Filippova, Moscow State University [8,9]. Spectra of the following proteolytic activities were tested using specific pNA substrates: cysteine proteinases from AM with pyroglutamyl-phenylalanyl-alanine pNA (GlpFAPNA), chymotrypsin-like proteinases from PM with pyroglutamyl-alanyl-alanyl-leucine pNA (GlpAALpNA), and trypsin-like enzymes also from PM with BzRpNA. The substrates were dissolved in dimethyl formamide at 20 mM and diluted to 1 mM with 0.1 M universal buffer [10], pH 5.6, containing 5 mM DTT (GlpFAPNA) or pH 7.9 (GlpAALpNA and BzRpNA).

Native PAGE. The electrophoretic resolution of active proteinases was performed in a native electrophoretic system according to McLellan [5] in a 1-mm-thick slab of 12% polyacrylamide gel with 35 mM Hepes and 43 mM imidazole running buffer, pH 6.8, 4°C . Each 4-mm² well contained 1.7 μl of extract, predetermined to hydrolyze 3–5 mM/min of nitroanilide substrate and corresponding to 30–60 μg of dialyzed protein as determined spectrophotometrically at 280 nm.

Detection of proteolytic activity. Activity detection was by a modified procedure of Ohlsson et al. [1]. After electrophoresis the gel was soaked for 15 min in an appropriate 0.1 M universal buffer. The buffer was removed, the residual buffer was removed with filter paper, and the gel was carefully covered by a nitrocellulose membrane (0.45 μm pore size, Amersham, Austria) presoaked for 40 min in the appropriate substrate solution and slightly air dried. The membrane in contact with the gel was incubated in a moist chamber at 37°C until faint yellow bands of the reaction product, nitroaniline, became visible on the membrane (usually 0.5–1 h). The gel was removed and liberated pNA was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% N -(1-naphthyl)ethylenediamine in 47.5% ethanol. Immediately after formation of pink

bands of proteolytic activity, membranes were placed in plastic bags, scanned, and stored at -20°C .

The proteolytic spectra obtained by the overlay technique were compared with the spectra obtained by electrotransfer of proteins to the nitrocellulose membrane according to Oppert et al. [2] using the same proteinase samples. For electrotransfer experiments, immediately after electrophoresis the appropriate part of the gel was covered by a nitrocellulose membrane and sheets of filter paper soaked in electrophoretic 35 mM Hepes and 43 mM imidazole buffer. Transfer was performed in a LKB Nova-Blot apparatus (LKB, Sweden) at 15 V and 250 mA for 0.5, 1, and 2 h at 25°C . The membrane was incubated in the appropriate substrate solution and the liberated pNA was diazotized as described above.

Inhibitor analysis. Specific inhibitors used in combination with the overlay technique of activity detection were inhibitors of cysteine proteinases, L -trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), serine proteinases, phenylmethylsulfonyl fluoride (PMSF), trypsin, N_{α} -tosyl-L-lysine chloromethyl ketone (TLCK), and soybean Kunitz trypsin inhibitor (STI). After PAGE, the gel was incubated in 0.05 mM E-64, 5 mM PMSF, 0.3 mM TLCK, or 0.02 mM STI solution in 10 mM phosphate buffer, pH 6.8, for 20 min at 25°C . In comparative experiments, concentrated inhibitor solution was added to the aliquot of proteinase extract before PAGE to the following final concentrations: 0.5 mM E-64, 5 mM PMSF, 1 mM TLCK, or 0.02 mM STI.

Results and discussion

The comparison of spectra of different proteolytic activities obtained using the overlay technique or electrotransfer are presented in Fig. 1. Cysteine proteinase activity (Fig. 1A) was visible only by means of the overlay technique and was lost during electrotransfer. This may be due either to detergent/heat denaturation or to an insufficient extent of transfer. Absence of the activity at the prolonged time intervals (1 and 2 h) used commonly for electrotransfer suggested that activity was

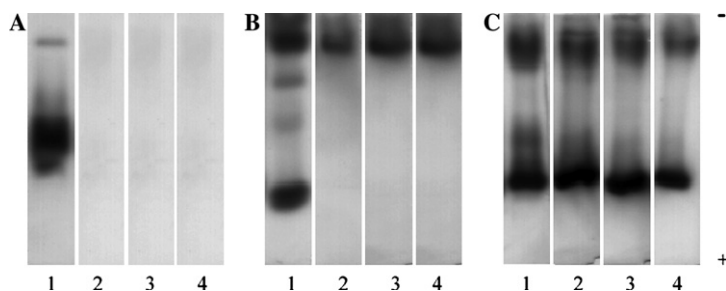


Fig. 1. Comparison of the spectra of proteolytic activities obtained by the overlay technique (lanes 1) and electrotransfer during 0.5 h (lanes 2), 1 h (lanes 3), and 2 h (lanes 4). (A) Activity of cysteine proteinases with GlpFAPNA at pH 5.6, 5 mM DTT. (B) Activity of trypsin-like proteinases with BzRpNA at pH 7.9. (C) Activity of chymotrypsin-like proteinases with GlpAALpNA at pH 7.9.

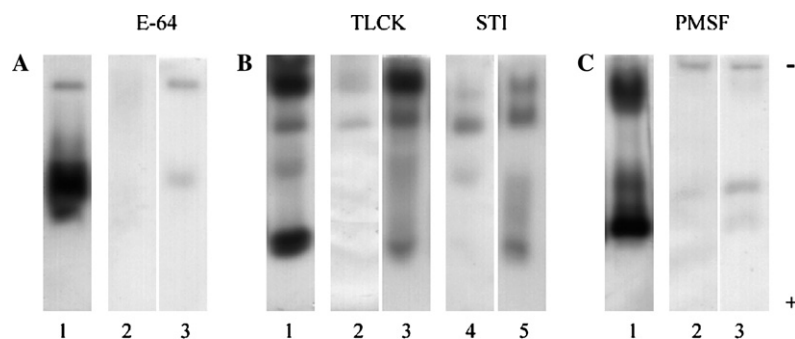


Fig. 2. Effect of inhibitors on the activity of proteinases after postelectrophoretic (lanes 2 and 4) and preelectrophoretic (lanes 3 and 5) incubation compared to the control without inhibitors (lanes 1). (A) Activity of cysteine proteinases with GlpFAPNA at pH 5.6, 5 mM DTT. (B) Activity of trypsin-like proteinases with BzRpNA at pH 7.9. (C) Activity of chymotrypsin-like proteinases with GlpAALpNA at pH 7.9.

lost due to denaturation, even at the 30-min time interval recommended for transfer of serine proteinases [4].

Serine proteinase activities were visible with the electrotransfer method (Figs. 1B and C). However, trypsin-like activity by means of the overlay technique was represented by a group of bands with low, intermediate, and high mobility, but after electrotransfer, only the low-mobility activity was observed (Fig. 1B). Chymotrypsin-like activity was detected by either method with similar spectra, except for a minor band with intermediate mobility that was absent in electrotransfer (Fig. 1C). Hence, the overlay technique, in contrast to electrotransfer, allowed for postelectrophoretic detection of the activity of unstable cysteine proteinases and extended the spectra of serine proteinases detected with specific pNA substrates. The minimum detection level for bovine trypsin was 40 μ M/min of liberated pNA for 0.1 μ g of enzyme (data not shown).

A comparison of two methods of inhibitor analysis of proteinase spectra demonstrated that incubation with inhibitors after electrophoresis was more effective, even when lower concentrations of inhibitors were used (for E-64 and TLCK) (Fig. 2). The differences in the extent of inhibition depended on the characteristics of the enzyme. E-64 added after PAGE completely inhibited the activity of cysteine proteinases, while a low-mobility band was insensitive to the inhibitor when added to the sample before PAGE at a 10-fold higher concentration (Fig. 2A). The effect of trypsin inhibitors TLCK and STI on trypsin-like activity was more evident with postelectrophoretic incubation, especially for fast- and slow-moving fractions (Fig. 2B). An inhibitor of serine proteinases, PMSF, added after PAGE completely inhibited chymotrypsin-like fractions except for the slowest migrating (Fig. 2C). However, preelectrophoretic incubation with PMSF resulted in additional incomplete inhibition of an intermediate migrating fraction.

Thus, application of the overlay technique for activity detection with specific chromogenic peptide substrates combined with postelectrophoretic inhibitor analysis permitted simple and detailed information on individual proteinases in complex mixtures. The improvement of

previously described transfer procedures resulted in enhanced detection of proteinases from several classes. We also demonstrated that the method of inhibitor incubation affected the characterization of specific proteinases in the mixture.

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